Introduction:

The worldwide need of energy is increasing day by day with expected reserve depletion of fossil fuels. The fossil fuels are considered to be the main energy source. It cannot be regenerated and will be exhausted with rapid consumption. Combustion of these fossil fuels, other pollutants and extractives has serious negative effects on environmental degradation, global climate change and health problems (Bockris, 2002). Fossil fuels releases CO_2, CH_4 and CO resulting in global warming and pollution which is major reason of global warming and climate change (Saratale *et al.,* 2008). For this reason various different alternative energy sources have been investigated which could substitute fossil fuel. Hydrogen (H_2) becomes a significant and alternate source of energy carrier with high energy yield (142 Kj/g) (Balat *et al*., 2008) to fossil fuels because it causes very little pollution (Cardoso *et al*., 2014). Hydrogen (H_2) gas is an ideal alternative to fossil fuels that does not evolve greenhouse gases considered universally, as clean energy source also environmental friendly (Al-Shorgani *et al*., 2014). It holds much promise as a dream fuel of the future, increasing requirement of energy sources using microorganisms. Also it is an efficient new area of technology that provides the potential for H_2 production from variety of renewable resources (Bhagat *et al*., 2014).

Hydrogen is being produced by various processes, mainly from natural gas, electrolysis of water, steam reforming of hydrocarbons and biological processes. A biological process depends upon the presence of hydrogen producing enzymes in microbes which is responsible for bio-hydrogen production. Biological hydrogen production can be classified into the following groups: 1) direct bio-photolysis, 2) indirect photolysis, 3) biological water-gas shift reaction, 4) photo-fermentation and 5) dark-fermentation and 6) hybrid system.

Direct bio-photolysis includes photosynthetic production of hydrogen from water that can convert solar energy into chemical energy in the form of H_2:

$$2H_2O + \text{light energy} \rightarrow 2\ H_2 + O_2$$

Green algae can utilize H_2 as an electron donor in the CO_2 fixation process or evolve H_2 under anaerobic condition (Ni *et al*., 2006).

Indirect bio-photolysis involves in synthesize and evolve H_2 through photosynthesis in two step process.

$$12H_2O + 6CO_2 + \text{light energy} \rightarrow C_6H_{12}O_2 + 6O_2$$

$$C_6H_{12}O_6 + 12H_2O + \text{light energy} \rightarrow 12H_2 + 6CO_2$$

Species of *Cyanobacteria* may possess several enzymes directly involved in hydrogen metabolism and synthesis of molecular H_2. Commonly used cyanobacterial species such as *Anabaena, Oscillatoria, Calothrix and Gloeocapsa* (Sen *et al*., 2008).

Biological water-gas shift reaction includes photohetrotrophic bacteria, such as *Rhodospirillum rubrum* can survive in dark using CO as the sole carbon source to generate ATP with release of H_2 and CO_2 (Levine *et al*., 2004).

$$CO + H_2O \rightarrow CO_2 + H_2, \Delta G^0 = -20 \text{ (Kj/mol)}$$

Photo-fermentation is based on decomposition of organic substrate to hydrogen in the absence of both O_2 and N_2 but in the presence of photosynthetic bacteria such as *Rhodobacter sphaeroides, Rhodopseudomonas capsulate, R. palustris, Rhodospirillum rubrum* etc. have potential to convert light energy into H_2 (Sen *et al*., 2008).

$$C_6H_{12}O_6 + 12H_2O + \text{light energy} \rightarrow 12H_2 + 6CO_2$$

Dark-fermentation process, hydrogen can be produced by anaerobic fermentative bacteria, grown in the dark on carbohydrate rich substrates. Fermentative bacteria can be mesophilic (25-40°C), thermophilic (40-65°C), extreme thermophilic (65-80°C) or hyperthermophilic (more than 80°C) temperatures (Levine *et al*., 2004). It produces a mixed biogas containing primarily H_2 and CO_2 but may also contain small amount of methane (CH_4), carbon monoxide (CO) and/or hydrogen sulphide (H_2S) depending on reaction process and substrate used. With glucose substrate, maximum 4 mol H_2 is produced per mole of glucose when end product is acetic acid:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$$

When the end product is butyrate, 2 mol of H_2 is produced:

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_2CH_2\ CH_2OOH + 2H_2 + 2CO_2$$

Bacteria which produce H_2 include species of *Enterobacter, Bacillus and Clostridium.* H_2 generation depends on many factors such as type and concentration of substrate, pH, hydraulic time of retention, substrate to inoculums ratio, metal ion concentration etc. Hydrogen can also be produced from proteins and lipid-rich substrates but carbohydrates are preferred because of higher yields (Levin *et al*., 2004; Claassen *et al*., 1999).

Hybrid system using fermentative and photosynthetic bacteria, the microbial production of hydrogen by fermentation can be broadly classified into two main categories. One category, light independent bacteria could provide an integrated system for maximizing the hydrogen yield.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \text{ (first stage-dark fermentation)}$$

$2CH_3COOH + 4H_2 \rightarrow 8H_2 + 4CO_2$ (second stage-photofermentation)

In such a system, the anaerobic fermentation of carbohydrate (or organic wastes) produces intermediates, such as low molecular weight organic acids, which are then converted into hydrogen by photosynthetic bacteria in the second step in a photo-bioreactor and total of 12 mol hydrogen could be expected in a combined process from one mol of glucose.

The enzymes catalyze the chemical reaction $2H^+ + 2e^- \leftrightarrow H_2$. At present three enzymes carrying out this reaction are depending on metal content at active site: nitrogenase, Fe-hydrogenase and NiFe-hydrogenase (Balat, 2009). Fe-hydrogenase enzyme is used in the biophotolysis processes wherea photo-fermentation processes utilize nitrogenase (Manish and Banerjee, 2007). Nitrogenases are MoFe protein and Fe protein has ability to use magnesium adenosine triphosphate (MgATP) and electrons to reduce a variety of substrates. Whereas hydrogenases; first, uptakes hydrogenases and second, reversible hydrogenases. Uptake hydrogeases, such as NiFe-hydrogenases and NiFeSe-hydrogenases that are used for catalysis of hydrogen consumption whereas reversible hydrogenases, have ability to produce H_2 as well as consume H_2 depending on the reaction condition.

Biomass is one of the important sources for bio-hydrogen production. Major biomass includes agricultural crops and their waste byproducts, wastes from food processing, aquatic plants and algae and effluents produced in human habitat. The main criteria for the selecting substrate for bio-hydrogen production are its availability, cost effective, carbohydrate content and their biodegradability (Das *et al*., 2008). Agricultural waste should be cut into small pieces or should be ground and then delignified by mechanical or chemical means before fermentation. Lignocelluloses residues have shown the possibility of using rice straw, rice husk, wheat straw and shorgham waste for energy generation (Chen *et al*., 2012). Rice based industry such as rice bran, rice husk, straw etc is used as substrate is one such agricultural residue that is produced abundantly as a by-product of rice crop with an annual worldwide production of 800 Mt (Wati *et al*., 2007). India is the second most country in the world and Chhattisgarh is fifth state in rice production. Hence agricultural waste percentage is also abundant therefore rice waste material can be used as substrate for bio-hydrogen production. Rice bran contains bound sugars in cellulose and hemicelluloses entangled with lignin moieties. It is by-product of rice milling industry, is obtained by the abrasive milling of brown rice to remove the outer coat cover which approximately 40 million metric tons are produced every year and are discarded as unfit for human consumption. Its main components

are carbohydrates, proteins, lipids, fibre and water. This residue, after extracting rice bran oil, is called de-oiled rice bran. Fermentative bacteria have very high evolution rate of hydrogen. Different bacterial strains are *E.coli, Clostridium butyricum* (Tanisho *et al*., 1987), *Enterobacter aerogenes* (Tanisho *et al.,* 1983) which can degrade complex carbohydrates. In proposed work, bacteria from byproduct of rice based industry will be isolated, identified and characterized by biochemical tests and molecular basis. Various parameters will be optimized for bio-hydrogen production via dark fermentation.

A brief review of work already done in the field:

Hydrogen production from different microorganisms is known as bio-hydrogen, offers various potential production of usable hydrogen from variety of renewable resources. Microalgae, both eukaryotic and prokaryotic bacteria have hydrogenases enzymes responsible for production of hydrogen under certain conditions. Tanisho *et al*., (1983) and Kumar and Das (2000), reported fermentative evolution of bio-hydrogen as most prominent method as photochemical evolution for mass production Nandi and Sengupta (1998) investigated hydrogen production with anaerobic bacteria during 1980s. Vatsala and Seshadri (1985) first reported the participation of microorganisms for efficient bio-hydrogen production. Benemann (1997) studied that of hydrogen by microorganisms. Woodward *et al.,* (2000) reported that a maximum of 11.6 mol H_2/mol of glucose is possible. Das and Veziroglu (2001) Levine *et al*., (2004) and Lin and Lay (2005) studied fermentative bio-hydrogen production processes which is greatly affected by many factors, such as pH, temperature, redox reaction potential and nutritional requirements. Fang *et al*., (2006) worked on bio-hydrogen production from rice slurry at 4.5 pH in batch fermentation using acidophilic bacteria. Kapdan and Kargi (2006) studied utilization of some carbohydrate rich, starch or cellulose containing substrate through combined bioprocess of dark and photo- fermentation as an attractive approach resulting in high hydrogen yields. Bacteria like *Enterobacter aerogenes* and *Clostridium buytricum* has been found to be efficient producers of bio-hydrogen, which has been reported by Tanisho and Ishiwata (1994), Yokoi *et al.,* (1998) and Nakashimada *et al.,* (2002). Biological hydrogen production by anaerobically digested sludge from corn-syrup using heat pre-treated can be achieved in the biohydrogenator was studied by Hafez *et al*., (2009). The hydrogen production ability of *Clostridium* strain AK14 a moderate thermophilic bacterium was studied by Almarsdottir *et al.,* (2010) and fermentation

of glucose resulted in the production hydrogen and carbon dioxide as well as acetate and ethanol. Ariunbaatar *et al.*, (2014) studied pretreatment methods to enhance anaerobic digestion of organic solid waste by various processes including mechanical, thermal, chemical and biological methods. Fermentative hydrogen production investigated by Wongthanate *et al.*, (2014) from food and beverage processing wastewater having pH (4.5 to 7.0), mesophilic (35°C ± 2°C), and thermophilic (50°C ± 2°C) temperature conditions and investigated the impacts of pretreatment on bio-hydrogen production.

Objectives:

- Sample collection- Waste of rice bran oil industry will be collected and bio-hydrogen producing bacterial strain will be seasonal periodically isolated.
- Characterization of isolated bacteria based on morphology, biochemical tests and their molecular identification.
- Optimization of different pretreatment methods (chemical and biological) for better production of bio-hydrogen.
- Optimization of different physical and chemical parameters for maximum bio-hydrogen production.

Noteworthy contribution in the field of proposed work:

Biological hydrogen production processes are mostly operated at ambient temperatures and pressures, thus less energy intensive and environmental friendly. Many anaerobic microorganism involved in bio-hydrogen production from carbohydrate containing organic wastes. *Clostridium* such as *C. buytricum* (Yokoi *et al.*, 2001), *C. Thermolacticum* (Collet *et al.*, 2004) *C. Pasteurianum* (Liu *et al.*, 2004 and Lin *et al.*, 2004), and *C. bifermentants* (Wang *et al.*, 2003) are obligate anaerobes and spore forming organisms required environmental conditions are provided for hydrogen production. Zhang *et al.*, (2003) studied the supplementation of starch waste water with vitamins and minerals could produce a biohydrogen yield of 92 mL /g starch in batch culture of *Thermoanaerobacterium* species. Bio-hydrogen production from untreated rice straw using different sludge, initial cultivation pH, concentration of the substrate and size of the particle was evaluated at 55 °C by Chen *et al.*, 2012. Bio-hydrogen production by fermentation of biomass with sterile and non sterile

substrate with mixed bacteria by Hussy *et al*., (2005). Akutsu *et al*., (2009) studied starch used as a sole substrate where continuous hydrogen production with a maximum hydrogen yield of 1.7 mol H_2/ mol glucose by using thermophilic granular sludge. Levin *et al.*, (2006) studied cellulose degrading hydrogen producer using *Clostridium thermocellum* for hydrogen production from cellulose-based medium with the yield of 1.6 mol H_2/mol glucose. Wang *et al*., (2008) investigated a co-culture of *Clostridium acetobutylicum* X9 and *Ethanoligenens harbinense* B49 in microcrystalline cellulose for hydrogen production. *E. harbinense* can produce hydrogen efficiently from monosaccharides but not cellulose. Al-Shorgani *et al*., (2014) studied various pretreated agroindustrial wastes, including rice bran, de-oiled rice bran, sago starch and palm oil mill effluent via *Clostridium saccharoperbutylacetonicum* N1-4 was investigated in a batch culture system at 30°C and a pH of 6.2.

Proposed methodology:

1. Sample collection and maintenance.
   Waste of rice bran oil industry will be collected in clean polybags.

2. Isolation and screening of fermentative bacterial strain.
   Bacterial strain from the substrate will be isolated on nutrient agar plates, by serial dilution method. Isolated bacterial strain will be then screened for biohydrogen production and its molecular identification will be carried out.

3. Effect of chemical pretreatments.
   Effect of acid and alkali pretreatment will be studied on bio-hydrogen production. Pretreatment will help in conversion of complex sugar into simpler which will be utilized by the bacteria.

4. Effect of biological pretreatments.
   Different biological agents such as bacteria and fungus will be inoculated in the substrate for pretreatments and its effect on bio-hydrogen will be studied.

5. Optimization of temperature and pH.
   Different temperature and pH will be optimized for better bio-hydrogen production.

6. Effect of inoculums size and age.
   Different inoculums age and size will be optimized for bio-hydrogen production.

7. Effect of static and stirred inoculums.
   Effect of stirring and static condition on bio-hydrogen production will be checked.

8. Effect of immobilization of microorganism. .
   Bio-hydrogen production will be carried out in two states of bacteria freely suspended and immobilized for efficient bio-hydrogen production.

9. Effect of metal ions.
   Effect of metal ions will be studied on bio-hydrogen production as metal ion act as enzyme cofactor and also effects cell growth.

10. Analysis of data.
    Data will be statistically analyzed by using desirable software.

Expected outcome of the proposed work:

Hydrogen is most promising alternative source of energy carriers in the future. Efficiency of bio-hydrogen processes will increase significantly by the removal of by-products while producing hydrogen. Biological dark fermentation is a most promising bio-hydrogen production method for commercial use in future. An efficient bio-hydrogen producing bacteria will be isolated. Substantial improvements, however, can be made through rapid gas removal and separation, bioreactor design, and genetic modifications in the microorganisms. Utilization of some carbohydrate rich substrate is an efficient approach for bio-hydrogen production. The biological and chemical pretreatment of lignocelluloses substrate under control of environmental conditions will help to improve fuel yields. A dark-fermentation system appears most prominent method to have the great potential to be developed as practical bio-hydrogen production systems.

References:

Akutsu, Y., Lee, D.Y., Chi, Y.Z., Li, Y.Y., Harada, H. and Yu, .Q.	2009	Thermophilic fermentative hydrogen production from starch-wastewater with bio-granules. *International Journal of Hydrogen Eenergy*, **34**:5061-5071.
Almarsdottir, A.R., Torazewich, A., Gunnarsson, I. and Orlygsson, J.	2010	Hydrogen production from sugars and complex biomass by *Clostridium* species, AK14, isolated from Icelandic hot spring. *Icelandic agricultural Sciences*, **23**:61-71.
Al-Shorgani, N.K.N., Tibin, El-M., Ali, E., Hamid, A.A., Yusoff, W.M.W. and Kalil, M.S.	2014	Biohydrogen production from agroindustrial wastes via *Clostridium saccharoperbutylacetonicum* N1-4(ATCC 13564), *Clean Technologies and Environmental Policy*, **16**:11–21.
Ariunbaatar, J., Panico, A., Esposito, G., Pirozzi, F. and Lens, P.N.L.	2014	Pretreatment methods to enhance anaerobic digestion of organic solid waste. *Applied Energy*, **123**:143-156.
Balat, M.	2009	Production of hydrogen via biological processe. *Energy Sources*, **31**:1802-1812.
Benemann, R.	1997	CO_2 mitigation with microalgae systems. *Energy Conversion and Management*, **31**: 683-692.
Bhagat, A.P., Shindikar, M. and Pethkar, A.V.	2014	Strategies for enhancement of biological hydrogen production. *Journal of Environmental Research And Development*, **8**:945-954.
Bockris, J.M.	2001	The origin of ideas on a hydrogen economy and its solution to the decay of the environment. *International Journal of Hydrogen Energy*, **27**:731-740.
Cardoso, V.L., Romao, B.B., Silva, F.T.M, Santos, J.G.,	2014	Hydrogen production by dark fermentation. *Chemical Engineering*

Batista, F.R.X. and Ferreira, J.S. *Transactions*, **38**:481-486.

Chen, C.C., Chuang, Y.S., Lin, C.Y. and Lay, C.H. 2012 Thermophilic dark fermentation of untreated rice straw using mixed cultures for hydrogen production. *International Journal of Hydrogen Eenergy*, **37**: 15540-15546.

Claassen, P.A.M., Van Lier, J.B., Lopez, C.A.M., Van, N.E.W.J., Sijtsma, L., Stams, A.J.M., Vires, S.S. and de Weusthuis, R.A. 1999 Utilization of biomass for the supply of energy carriers. *Applied Microbiological Biotechnology*, **52**, 741-755.

Collet, C., Adler, N., Schwitzguebel, J.P. and Peringer, P. 2004 Hydrogen production by *Clostridium thermolacticum* during continuous fermentation of lactose. *International Journal of Hydrogen Energy*, **29**:1479–85.

Das, D. and Veziroglu, T.N. 2001 Hydrogen production by biological processes a survey of literature. *International Journal of Hydrogen Energy*, **26**:13-28.

Das, D. and Veziroglu, T.N. 2008 Advances in biological hydrogen production processes. *International Journal of Hydrogen Energy*, **33**: 6064-6065.

Fang, H.H.P., Li, C. and Zhang, T. 2006 Acidophilic biohydrogen production from rice slurry. *International Journal of Hydrogen Energy*, **31**:683-692.

Hafez,H., Kakhla, G. and El-Naggar, H. 2009 Biological hydrogen production from corn syrup waste using a novel system. *Energies*, **2**: 445-455.

Hussy, I.K., Hawkes, R., Dinsdale, R. and Hawkes, L. 2005 Continuous fermentative hydrogen production from sucrose and sugar beet. *International Journal of Hydrogen*

Energy, **30**: 471-483.

Kapdan, I.K. and Kargi, F. 2006 Bio-hydrogen production from waste materials. *Enzyme and Mirobial Technology*, **38**:569-582.

Kumar, N. and Das, D. 2000 Enhancement of hydrogen production by *Enterobacter cloacae* IIT-BT 08. *Process Biochemistry*, **35**:589-593.

Levin, D.B., Islam, R., Cicek, N. and Sparling, R. 2006 Hydrogen production by *Clostridium thermocellum* 27405 from cellulosic biomass substrates. *Internation Journal of Hydrogen Eenergy*, **31**:1496-1503.

Levin, D.B., Pitt, L. and Love, M. 2004 Biohydrogen production: prospects and limitations to practical application. *International Journal of Hydrogen Energy*, **29**:173-185.

Lin, C.Y. and Lay, C.H. 2005 Anutrient formulation for fermentative hydrogen production using anaerobic sewage sludge microflora. *International Journal of Hydrogen Energy*, **30**:285-292.

Lin, C.Y. and Lay, C.H. 2004 Carbon/nitrogen ratio effect on fermentative hydrogen production by mixed microflora. *International Journal of Hydrogen Energy*, **29**: 41–5.

Liu. G. and Shen, J. 2004 Effects of culture medium and medium conditions on hydrogen production from starch using anaerobic bacteria. *Journal of Bioscience and Bioeng*ineering, **98**:251–256.

Manish, S. and Banerjee, R. 2008 Comparison of biohydrogen production processes. *International Journal of Hydrogen Energy*, **33**:279-286.

Nakashimada, Y., Rachman, M.A., Kakizono,T. and Nishio, N.	2002	Hydrogen production of *Enterobacter aerogenes* altered by extracellular and intracellular redox states. *International Journal of Hydrogen Energy*, **27**: 1399-1405.
Nandi, R. and Sengupta, Y.	1998	Microbial production of hydrogen: an overview. *Critical Review in Microbiology*, **24**:61-84.
Ni, M., Leung, D.Y.C., Leung, M.K.H. and Sumathy, K.	2006	An overview of hydrogen production from biomass. *Fuel Processing Technology*, **87**:461-472.
Saratale, G.D., Chen, S.D., Lo, C.Y., Saratale, R.G. and Chang J.S.	2008	Outlook of biohydrogen production from lignocellulosic feedstock using dark fermentation- a review. *Journal of Scientific & Industrial Research;* **67**:962-979.
Sen, U., Shakdwipee, M. and Banerjee, R.	2008	Status of biological hydrogen production. *Journal of Scientific & Industrial Research*, **67**:980-993.
Tanisho, S., Suzuki, Y. and Wakoo, N. .	1987	Fermentative hydrogen evolution by *Enterobacter aerogenes* strain E.82005. *International journal of Hydrogen Energy*, **12**: 623-670.
Tanisho, S. and Ishiwata. Y.	1994	Continuous hydrogen production by molasses by bacterium *Enterobacter aerogenes. International Journal of Hydrogen Energy*, **19**:807-812.
Tanisho, S., Wakoo, N. and Kokako, Y.	1983	Biological hydrogen production by *Enterobacter aerogenes, Journal of Chemical Engeneering Japan*; **16**: 529-530.

Vatsala, T.M. and Seshadri, C.V.	1985	Microbial production of hydrogen-a review. *Proceeding of the Indian National Science Academy*, **51**: 282-295.
Wang, C.C., Chang, C.W., Chu, C.P., Lee, D.J., Chang, B.V. and Liao, C.S.	2003	Producing hydrogen from wastewater sludge by *Clostridum bifermentans. Journal of Biotechnology*, **102:**83-92.
Wang, A., Ren, N., Shi, Y. and Lee, D.J.,	2008	Bioaugmented hydrogen production from microcrystalline cellulose using co-culture – *Clostridium acetobutyricum* X9 and *Ethanoligenens harbinense* B49. *Internation Journal of Hydrogen Eenergy*, **33**:912-7.
Wati, L., Kumari, S. and Kundu, B.S.	2007	Paddy straw as substrate for ethanol production. *Indian Journal of Microbiology*, **47**:26-29.
Wongthanate, J., Chinnacotpong, K. and Khumpong, M.	2014	Impacts of pH, temperature, and pretreatment method on biohydrogen production from organic wastes by sewage microflora. *International Journal of Environmental Engineering*, **5**:1-6.
Woodward, J., Orr, M., Cordray, K. and Greenbaum, E.	2000	Enzymatic production of biohydrogen. *Nature*, **405**: 1014-1015.
Yokoi, H., Tokushige, T., Hirose, J., Hayashi, S., and Takasaki, Y.	1989	H_2 production from starch by a mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes. Biotechnology Letters*, **20**: 1-143.
Yokoi. H., Saitsu, A.S., Uchida, H., Hirose, J., Hayashi, S. and Takasaki, Y.	2001	Microbial hydrogen production from sweet potato starch residue. *Journal of Bioscience and Bioeng*ineering, **91**:58-63.

Zhang, T., Liu, H. and Fang, H.H.P. 2003 Biohydrogen production from starch in wastewater under thermophilic condition. *Journal of Environmental Management*, **69**:149-156.

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